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13. ABSTRACT *(Maximum 200 words)*

The role of DNA damage intermediates and the network of signalling pathways by which cells activate p53 in the overall response to DNA damage is not well defined. It is known that p53 binds DNA in a sequence-specific manner as well as DNA containing insertion/deletion mismatches (IDLs). In the funded studies, we determined that p53 requires intact central and dimerization domains to complex with IDLs and that the K_D of p53 binding to IDLs is 45 pM as compared to a K_D of 31 pM for p53 binding to DNA fragments containing a consensus binding site. The picomolar dissociation constant that we observed for p53 binding to IDLs suggests that p53 would bind to IDLs in the cell. To investigate this possibility, oligonucleotide duplexes containing an IDL lesion were transfected into cells and the cells crosslinked to "trap" p53/DNA complexes. Our crosslinking-based technique has also allowed us to investigate the kinetics of p53 binding to downstream target gene promoters (e.g. $p21^{waf1}$, mdm2, and PIG3) and determine whether specific post-translational modifications of p53 are required for transactivation. Understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

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Suzanne T. Szak
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Introduction

The most critical property of the p53 tumor suppressor protein is probably its DNA binding activity. This hypothesis is supported by the fact that the vast majority of all tumor-derived mutant forms of the protein have point mutations in the central DNA binding domain, abrogating the ability of p53 to bind DNA in a sequence-specific manner. Furthermore, the ability of p53 to function as a transcriptional activator is believed to be integral for its growth suppressive properties. Therefore, a thorough understanding of the p53-DNA interaction is key for gaining insight to the biochemistry of this tumor suppressor protein. With the research support provided by this training grant, I have analyzed the interaction of the transcription factor p53 with DNA lesions and its consensus DNA binding site in downstream promoters. The model lesion used in this study was the insertion/deletion mismatch (IDL). Initially, *in vitro* studies were performed to map the domain of p53 required for IDL binding and to compare the affinity of p53 for its consensus site and the IDL. My results indicate a high affinity for p53 binding to an IDL and its consensus site; therefore, I am currently trying to detect p53 bound to IDLs in cells using a formaldehyde crosslinking protocol. This technique is also allowing me to study the kinetics of p53 binding to various endogenous downstream target gene promoters in control cells versus cells that have undergone genotoxic stress. The promoters under investigation include $p21^{waf1}$, mdm2, and PIG3. The results of this study will elucidate some aspects of the mechanism of regulation of p53 transactivation, including whether p53 is costitutively docked at downstream target promoter sites and if acetylation, one of the post-translational modifications of p53 shown to enhance its DNA binding activity *in vitro*, is required for its transactivation ability in cells. Ultimately, determining if p53 is directly activated by DNA damage intermediates and understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

Body

In vitro Binding Studies

In addition to binding DNA in a sequence-specific manner, p53 binds to insertion-deletion lesions (IDLs). My studies show that the central domain of p53 is required for p53 binding to IDLs. Tumor-derived mutant forms of p53 lost IDL binding and C-terminal truncation mutants still bound IDLs if the protein maintained dimerization capability. From saturation binding studies, I determined that the K_D of p53 binding to IDLs is 45 pM, and the K_D of p53 binding to its consensus site is 31 pM. Consistent with these dissociation constants, p53-IDL complexes are dissociated with relatively low concentrations of competitor consensus site-containing DNA. The picomolar dissociation constant that we report for p53 binding to IDLs is consistent with the physiological concentrations of both p53 and DNA lesions in the cell after DNA damage. To investigate if p53 binds to IDLs in cells, phosphorothioate oligonucleotide duplexes containing an IDL lesion and a 5' biotin group are being transfected into cells and the cells treated with formaldehyde to crosslink or "trap" p53/DNA complexes. Transfected oligonucleotides can then be recovered using a streptavidin matrix. If p53 proteins were bound to the oligonucleotides containing IDLs, they will also be recovered on the streptavidin matrix.

The interaction of p53 with DNA damage intermediates may constitute an upstream event that triggers the activation and stabilization of the p53 protein. However, the role of DNA damage intermediates and the network of signalling pathways by which cells activate p53 in the overall response to DNA damage is not well defined. Determining if p53 is directly activated by DNA damage intermediates and understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

In vivo Binding Studies

Most studies of p53 transactivation have been performed using *in vitro* assays or in cells expressing exogenous reporter genes. These reports have not addressed fundamental questions regarding the regulation of the transactivating ability of p53. Specifically, it has not been determined whether p53 is bound to promoter sites continuously or in a transitory manner. Also, many recently reported post-translational modifications of p53 are hypothesized to be required for DNA binding. Furthermore, the mechanism dictating the selectivity of p53 for different promoters is unknown. That is, the expression of p53 target gene products must be coordinated according to the needs of the cell. For example, it is assumed that p53 is constitutively active for binding at the

mdm2 promoter since the mdm2 protein regulates the levels of p53 in the setting of a normal cell. On the other hand, p53 must be activated, perhaps by a post-translational modification, or merely present above a certain threshold level, in order to upregulate the expression of the cyclin-dependent kinase inhibitor, $p21^{waf1}$.

In order to study p53-DNA interactions in the context of an intact cell, I have developed an *in vivo* crosslinking procedure that enables me to "trap" p53/DNA complexes in the cell. By trapping p53 *in vivo*, many aspects of its regulation can be addressed. In order to determine whether a critical step of p53 transactivation occurs at a DNA binding or a post-DNA binding step, I will crosslink cells with formaldehyde, and any DNA to which p53 is bound will be immunoprecipitated and PCR amplified. In this way, the kinetics of p53 occupancy at endogenous promoters of downstream genes, such as $p21^{waf1}$, mdm2, and PIG3, can be evaluated in untreated cells and cells that have been genotoxically stressed. Furthermore, it can be determined whether acetylation, a post-translational modification of p53, is required for DNA binding and transactivation.

p53 can be acetylated by p300 at its carboxyl terminus. This acetylation event increases p53 sequence-specific DNA binding *in vitro*, suggesting that acetylation may be an upstream event of p53 activation. Furthermore, by using antibodies specific for the acetylated p53, Sakaguchi *et al.* have shown an increase in the levels of the acetylated forms of p53 in cells following gamma and ultraviolet radiation. Whether acetylation represents a level of regulation of p53 or is a basal post-translational modification remains unclear. Through a collaboration with CalBiochem, I have access to antibodies which specifically recognize and immunoprecipitate an acetylated form of p53 (Lys 382). This reagent is being used to determine the acetylation status of p53 at specific promoters.

The goal of the latter part of this study is to determine the kinetics of promoter occupancy by p53 and whether this DNA binding is dictated by the acetylation state of p53. Furthermore, since some p53 transcriptional targets have more than one p53 response element in their promoters, the specific site to which p53 is bound can be assessed. Functional consequences can thus be linked to p53 binding to specific sites in a promoter. Up to now, I have determined that, in agreement with protein analyses, p53 occupancy at the $p21^{waf1}$ promoter is an early event following genotoxic stress, whereas its binding to the PIG3 promoter occurs much later. The p53 docked at these promoters is acetylated, and studies are currently underway to assess the

approximate ratio of acetylated p53 to unacetylated p53 bound to these promoters.

STATEMENT OF WORK--Progress and Pitfalls

Specific Aim 1. To characterize p53 binding to both sequence-specific and non-sequence-specific DNA fragments.
COMPLETE

Specific Aim 2. To determine if other proteins modulate the p53/DNA interaction.

Months 1-2: Optimization of p53 running conditions for Surface Plasmon Resonance (SPR) on the Pharmacia BIACore instrument in the Molecular Recognition Core Lab at Vanderbilt University.

Months 1-3: Optimization of XPA growth and purification conditions

Months 4-6: Optimization of XPA conditions on the BIACore instrument.

Month 7: Comparison of XPA and p53 binding to C_3 DNA fragment.

Months 8-10: DNA binding experiments on the BIACore using RPA.

Richard Wood (Imperial Cancer Research Fund) provided me with a plasmid for XPA protein expression. I was able to optimize XPA expression in BL21 cells and purify it with good yield and the purity.

Initially I was able to detect p53 binding to consensus site DNA by using SPR. In addition, I observed RPA binding to the C_3 DNA; however, RPA yielded such a large signal that it was difficult to detect a difference with and without p53. In fact, despite extensive troubleshooting with buffer conditions and running parameters (i.e. flow rate, flow time, protein concentration), I was never able to detect p53 binding the C_3 fragment using SPR. Furthermore, after just a few weeks, I was no longer able to detect any p53-DNA binding. The streptavidin-coated chips which are used for this technology are supposed to maintain activity for up to approximately 100 runs. With the failure of this key reagent, this experimental approach was not considered feasible. My priority therefore shifted to Specific Aim 3.

Specific Aim 3. To determine the effect of post-translational modification on p53 DNA binding affinity.

Months 1-6: Assay p53 half-life in a DNA (consensus site and C_3) bound vs. unbound state using an *in vitro* degradation assay.

Months 7-9: Optimize assay conditions for acetylating p53 *in vitro* with the HAT domain of p300.

Months 10-12: DNA binding experiments using acetylated p53.

My preliminary studies showed that p53 bound to C_3 does not offer protection from E6-mediated degradation. However, proof of p53 bound to DNA was required. In order to do so, and to obtain a reagent to allow for detection of p53 bound to the C_3 lesion in cell culture, I employed the use of biotin end-labelled phosphorothioate oligonucleotides. In this way, p53 bound to the DNA can be isolated with streptavidin beads. Unfortunately, I found that p53 *in vitro* (and every protein, for that matter) binds non-specifically to DNA with this altered backbone due to its polyanionic nature.

I received a plasmid from Robert Roeder (Rockefeller University) for the expression of the HAT domain of p300, and I optimized the purification of active acetyltransferase activity from BL21 cells. I was able to acetylate p53 protein purified from bacteria using this enzyme and ^{14}C-labelled acetyl co-enzyme A. The efficiency of p53 acetylation was much less than that of histone acetylation. Through a collaboration with CalBiochem, I gained access to antibodies specific for p53 acetylated on Lysine 382 which provides a much more sensitive readout of p53 acetylation. This antibody is enabling me to study the acetylation state of p53 at endogenous target promoters in cells as described in the Body of this Annual Report.

Key Research Accomplishments

- The p53 tumor suppressor protein requires an intact central domain and dimerization in order to bind insertion-deletion lesions in DNA.

- The K_D of p53 binding to IDLs is 45 pM as compared to a K_D of 31 pM for p53 binding to DNA fragments containing a consensus binding site. These affinities are in the physiological range.

- The p53-IDL complex is efficiently dissociated with relatively low concentrations of consensus site DNA, but the binding of p53 to the consensus site is stable, even in the presence of excess IDL DNA.

- Acetylated p53 is detectable at specific downstream target promoters in a control population of cells, and its occupancy increases following genotoxic stress.

- p53 docks at the $p21^{waf1}$ and mdm2 promoters at early timepoints following genotoxic stress, but its occupancy at the PIG3 promoter is not apparent until approximately a 24 hour timepoint.

Reportable Outcomes for Grant DAMD17-97-1-7267

Degree

1994-present — Vanderbilt University School of Medicine, Nashville, TN
Dissertation Research in the Department of Biochemistry
• Analysis of the p53 Tumor Suppressor DNA Binding Activity
Advisor: Jennifer A. Pietenpol, Ph.D.

Awards

1998 — Leon Cunningham Graduate Biochemistry Award

1997 & 1998 — First Place Poster Award, Vanderbilt Cancer Center Retreat

Publications

Szak, S.T. and Pietenpol, J.A., "High Affinity Insertion/Deletion Lesion Binding by p53: Evidence for a Role of the p53 Central Domain." J. Biol. Chem. **274**, 3904-3909, 1999.

Szak, S.T., Pietenpol, J.A., and Carbone, D.P., "p53 and Human Lung Cancer," in *Lung Cancer: Principles and Practice* edited by Harvey I. Pass, James B. Mitchell, David H. Johnson, and Andrew T. Turrisi. Lippincott-Raven Publishers, Philadelphia. In preparation.

Meetings

1998 — Keystone Symposia, "The Cell Cycle", Keystone, CO
Poster Presentation

Vanderbilt Biochemistry Retreat
1996 — Oral Presentation
1998 — Poster Presentation

1997, 1998, 1999 — Vanderbilt Cancer Center Retreat
Poster Presentations

Resulting Research Opportunity

Starting November, 1999:

Applied for and received a post-doctoral research position with Mark Boguski at the National Center for Biotechnology Information (NCBI)

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High Affinity Insertion/Deletion Lesion Binding by p53

EVIDENCE FOR A ROLE OF THE p53 CENTRAL DOMAIN*

(Received for publication, June 12, 1998, and in revised form, October 8, 1998)

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In addition to binding DNA in a sequence-specific manner, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind short single-stranded DNA and double-stranded DNA containing nucleotide loops; these diverse associations may be critical for p53 signal transduction. In this study, we analyzed p53 binding to DNA fragments containing insertion/deletion mismatches (IDLs). p53 required an intact central domain and dimerization domain for high affinity complex formation with IDLs. In fact, the C terminus of p53 (amino acids 293–393) was functionally replaceable with a foreign dimerization domain in IDL binding assays. From saturation binding studies we determined that the K_D of p53 binding to IDLs was 45 pM as compared with a K_D of 31 pM for p53 binding to DNA fragments containing a consensus binding site. Consistent with these dissociation constants, p53-IDL complexes were dissociated with relatively low concentrations of competitor consensus site-containing DNA. Although p53 has a higher affinity for DNA with a consensus site as compared with IDLs, the relative number and availability of each form of DNA in a cell immediately after DNA damage may promote p53 interaction with DNA lesions. Understanding how the sequence-specific and nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how signaling cascades are initiated after DNA damage.

Exposure of normal cells to agents that damage DNA initiates a p53 signal transduction cascade, resulting in either cell cycle arrest or apoptosis (1). The interaction of p53 with DNA is thought to be critical for its signaling because the majority of tumor-derived forms of p53 have mutations in the central DNA binding domain (2), abrogating the ability of p53 to bind its consensus DNA sites (3). To date, many studies have focused on the sequence-specific interaction of p53 with DNA, including those that have identified downstream transcriptional targets and studies that describe post-translational modifications that activate p53 consensus site binding.

The ability of p53 to function as a transcriptional activator is believed to be integral for its growth-suppressive properties (4, 5). Sequence-specific transactivation is one of the most well understood biochemical activities of p53. After cellular stress such as DNA damage (6), hypoxia (7), viral infection (8), or activation of oncogenes such as *ras* (9) and *myc* (10), p53 becomes transcriptionally active. Once active, p53 induces, among many genes, p21 (11), an inhibitor of cyclin-dependent kinases thought to be necessary for the p53-dependent G_1/S cell cycle arrest (12–15). The increase in p53-mediated transcriptional activity may be because of elevated levels of p53 in the cell (6, 16) or increased sequence-specific binding ability (17, 18). Post-translational modifications of p53, including phosphorylation by S and G_2/M phase cyclin-dependent kinase-cyclin complexes (19, 20), DNA-dependent protein kinase (21), protein kinase C (22), and casein kinase II (23), as well as C-terminal acetylation (24) have been found to enhance sequence-specific DNA binding *in vitro*. Furthermore, p53 has been shown to be acetylated at its C terminus after exposing cells to ultraviolet or ionizing radiation (18). This acetylation may be regulated by phosphorylation of the p53 N terminus by either the ATM kinase or DNA-dependent protein kinase (18, 21, 25, 26).

In addition to binding DNA containing consensus sites, p53 can interact with nucleic acids in a sequence-independent manner. p53 can bind RNA (27), short single-stranded DNA (ssDNA)[1] (28–30), and double-stranded DNA containing nucleotide loops (31); these diverse associations may be critical to p53 signal transduction. The ability of p53 to bind ssDNA is of interest because this form of DNA is an intermediate of both DNA damage and repair. Studies have correlated p53 signaling activation with both the timing and amount of DNA strand breaks. Nelson and Kastan (32) have shown that p53 levels increased after electroporation of enzymatically active restriction endonucleases into cells. Microinjection of single-stranded circular phagemid or circular DNA with a large gap into nuclei of normal human fibroblasts induced a p53-dependent G_1 arrest (33). Jayaraman and Prives (34) reported stimulation of p53 consensus site binding *in vitro* in the presence of short ssDNA fragments. Further proof that p53 may directly interact with damaged DNA was provided in a study reporting p53 binding to DNA fragments containing insertion/deletion lesions (31). Also, confocal microscopy studies have shown colocalization of p53 protein with sites of damaged DNA in histological sections of human skin exposed to UV light (35). Collectively, these reports suggest that p53 may be directly or indirectly regulated by DNA damage intermediates. A direct interaction of p53 with either DNA lesions or with proteins that bind damage intermediates may be a relevant upstream event in the biochemical engagement of the protein.

In this study, we analyzed the interaction of p53 with DNA fragments containing insertion/deletion lesions. In contrast to

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; wt, wild-type; IDLs, DNA fragments containing insertion/deletion lesions; PAGE, polyacrylamide gel electrophoresis; CC, coiled-coil; NER, nucleotide excision repair.

many studies that have shown p53 C-terminal binding to ssDNA or DNA lesions, we demonstrate that an intact central domain and dimerization capability are required for wild-type (wt) human p53 binding to IDLs. The results of our binding analyses demonstrate that the affinity of p53 for DNA fragments containing either an IDL or a consensus site is in the pM range. Competition binding assays revealed that p53-IDL complexes were dissociated with relatively low concentrations of consensus site-containing DNA. However, the number and availability of each DNA site immediately after DNA damage may promote p53 binding to DNA lesions in lieu of sequence-specific DNA binding.

EXPERIMENTAL PROCEDURES

Expression of p53 Proteins—Sf9 cells were infected with either wt p53 or mutant $p53^{273}$-expressing recombinant baculovirus (kindly provided by C. Prives, Columbia University). Protein extracts of infected cells were harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 1 μM of E-64, antipain (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml; Sigma), and 4(2-aminoethyl)benzenesulfonylfluoride (200 μg/ml; Calbiochem-Novabiochem Corp.)) and sonicated, and extracts were incubated on ice for 30 min. The protein lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatant was collected and stored at −80 °C.

Extracts from Sf9 cells infected with baculoviruses expressing various forms of histidine-tagged murine p53 (generously provided by P. Tegtmeyer, State University of New York) were prepared as outlined, above except the lysis buffer used was DNA Binding Buffer (DBB: 20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM dithiothreitol).

p53 expression vectors (4) were transformed into *Saccharomyces cerevisiae* strain YPH681. Expression was induced by substituting galactose for dextrose in liquid cultures. Protein extracts were obtained by adding DBB and glass beads to the yeast pellets. Yeast were lysed using a bead beater, and extracts were clarified by centrifugation at 12,000 × *g* for 10 min at 4 °C.

DNA Fragments—The following oligonucleotides were used; underlined sequences represent the triplet cytosines. $3C_3$, 5′-CGAACCCGTTCTCGGAGCACCCCTGCCCCAGCCCAACCGCTTTGGCCGCCGCCCAGCC-3′; C_3, 5′-CGAACCCGTTCTCGGAGCACTGCCCCAGAACCGCTTTGGCCGCCGCCCAGCC-3′; negative control random sequence: 5′-CGAACCCGTTCTCGGAGCACTGCAGAACCGCTTTGGCCGCCGCCCAGCC-3′; consensus binding site (nucleotides 2293–2332 of the p21 promoter (11), the consensus site is underlined): 5′-TGGCCATCAGGAACATGTCCCAACATGTTGAGCTCTGGCA-3′. Oligonucleotides were purified on 10% PAGE/1× Tris borate-EDTA, 7 M urea gels, and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England BioLabs). Complimentary DNA strands were then annealed, and duplexes were purified using 10% PAGE with 1× Tris acetate/borate-EDTA.

DNA Binding Assay—To study the p53-IDL interaction, we used an *in vitro* protein-DNA binding assay developed by McKay (36) that allows for quantitative analysis. A monoclonal antibody that recognizes the N terminus of p53, PAb1801, was chemically cross-linked to protein A-Sepharose (PAS) with 52 mM dimethyl pimelimidate (Pierce); this antibody does not interfere with the oligomerization or DNA binding ability of p53. For assays using murine p53 (N-terminally tagged with six histidine residues), a monoclonal Penta-His antibody (Qiagen) was cross-linked to Protein G-Sepharose (PGS). The antibody-PAS/PGS complex was added to yeast or baculoviral protein extracts and mixed end-over-end for 1.5 h at 4 °C in 250 μl of DBB. Immunoprecipitated p53 was washed once with DBB, followed by a 5 min end-over-end wash with 0.5 M NaCl in Buffer B (5X Buffer B contains: 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 50% glycerol), and finally by a 5 min end-over-end wash in DBB. Subsequent analysis of these samples by SDS-PAGE and silver staining showed immunopurification of p53 to ~95–98% homogeneity. The immunopurified protein was then mixed end-over-end for one h with 3.5 fmoles of [^{32}P]-end-labeled DNA fragments in 250 μl of DBB for 1 h at room temperature. After three washes with DBB, protein components of the complex were digested with SDS/Proteinase K (VWR Scientific) in TE8 (20 mM Tris pH 8.0, 10 mM EDTA). DNA was phenol-chloroform extracted, ethanol precipitated, and electrophoresed in 1× Tris-acetate/EDTA on a 10% PAGE at 100 V. Gels were fixed in 5% methanol, 5% acetic acid before drying and exposure to film. DNA was quantified using a 445 SI PhosphorImager (Molecular Dynamics) and an Instant Imager (Packard Instruments). Alternative processing of the protein-DNA complexes for protein detection involved adding Laemmli sample buffer to the final complex and subjecting samples to 10% SDS-PAGE electrophoresis. Gels were stained with GelCode blue stain reagent (Pierce).

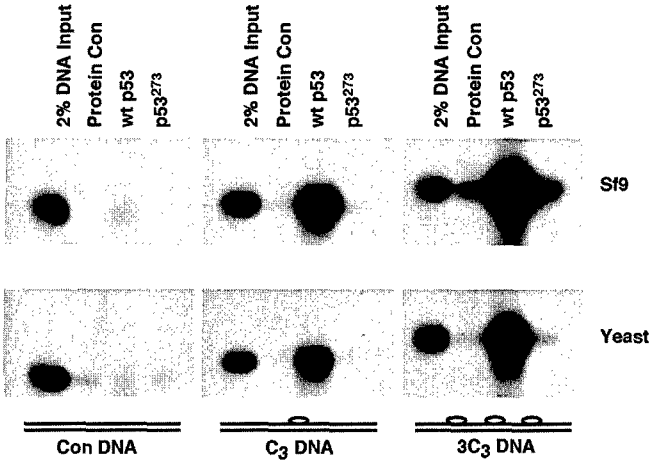

FIG. 1. **p53 binds DNA containing insertion/deletion mismatches.** Binding assays were performed with the DNA fragments shown and p53 protein preparations from Sf9 or yeast cells. Control DNA (*Con DNA*) represents a random sequence, double-stranded DNA fragment. The DNA fragments containing IDLs were formed by inserting either three extra cytosines (C_3) or three sets of three extra cytosines ($3C_3$) into one of the DNA strands. Protein control (*Protein Con*) represents an immunoprecipitate of a crude protein lysate from Sf9 or yeast cells not engineered to express p53 protein. $p53^{273}$ represents the Arg → His^{273} mutant. An equivalent amount of p53 protein was used in each assay, and the results are representative of five independent experiments.

Saturation Binding Assays—DNA binding assays were performed as outlined above using 0.1 pmol of wt p53 protein immunopurified from Sf9 cells and the indicated amounts of consensus or C_3 DNA fragment. For each DNA input, the nonspecific binding component was determined by performing side-by-side assays with the PAb1801-protein A-Sepharose complex incubated with protein from mock-infected Sf9 cells. After final washes, the tubes were counted in a scintillation counter.

Competition Binding Assays—DNA binding assays were performed as outlined above. The equilibrium DNA binding condition of C_3 was used (0.1 pmol of p53, 22 pM C_3 DNA). After counting the initial bound cpm, radiolabeled C_3 fragment bound to p53 was competed by the addition of the indicated amounts of unlabeled DNA in 250 μl of DBB. After 1 h of incubation at room temperature, complexes were washed three times with DBB, and the tubes were counted in a scintillation counter.

Prism software (GraphPad) was used for analyses of binding data. The percent incorporation of each radiolabeled DNA fragment, the nonspecific binding component, and radioactive decay rate of the DNA fragments were corrected for in the analyses.

RESULTS

To study p53-IDL interactions, we used an *in vitro* protein-DNA binding assay developed by McKay (36) that allows for quantitative analysis. The DNA lesions in this study were the same that Lee *et al.* (31) used to demonstrate p53 binding to IDLs through gel shift and electron microscope analyses. Random sequence, double-stranded DNA fragments with either one (C_3) or three ($3C_3$) sets of triplet cytosines in one of the strands were used; the extra bases caused a nucleotide loop in the DNA duplex.

Full-Length p53 Binds to DNA Fragments Containing IDLs—p53 protein produced in either baculoviral or yeast overexpression systems bound to DNA fragments containing IDLs at a level proportional to the number of triple cytosine loops in the DNA (compare *middle* and *right panels* of Fig. 1). Comparable p53 binding activity was also seen using DNA fragments of different sequence context that contained a triple cytosine

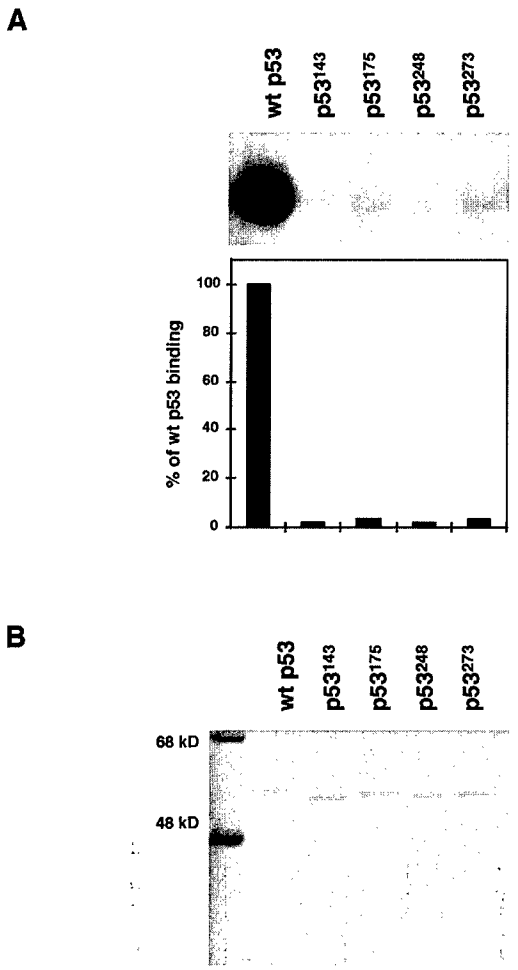

FIG. 2. **Tumor-derived mutant p53 proteins lack C_3 binding activity.** *A*, the results of binding assays performed with the C_3 DNA fragment and protein immunopurified from yeast cells engineered to express the following p53 point mutants: $Val^{143} \rightarrow Ala$, $Arg^{175} \rightarrow His$, $Arg^{248} \rightarrow Trp$, and $Arg^{273} \rightarrow His$. Quantification of the bound radiolabeled DNA is presented in the histogram. *B*, level and purity of the proteins used in the assay as visualized on a Coomassie Blue-stained SDS-polyacrylamide gel. Results are representative of five independent experiments.

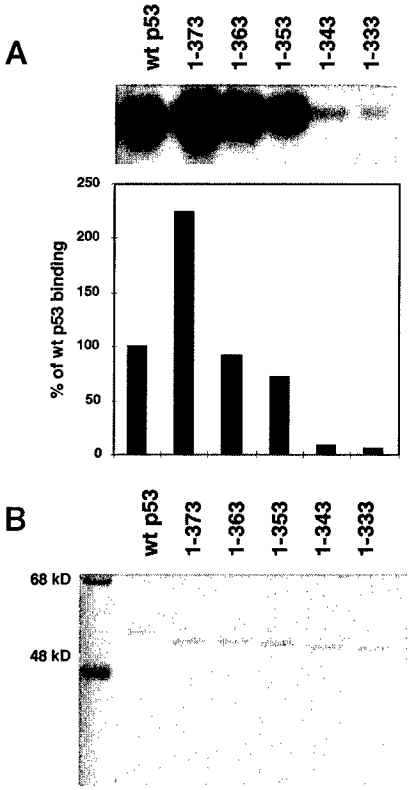

FIG. 3. **Deletion of the C-terminal 40 amino acids of p53 abrogates C_3 binding.** *A*, the results of binding assays performed with the C_3 DNA fragment and C-terminal-truncated p53 proteins immunopurified from yeast cells. Quantification of the bound radiolabeled DNA is presented in the histogram. *B*, level and purity of the proteins used in the assay as visualized on a Coomassie Blue-stained SDS-polyacrylamide gel. Results are representative of five independent experiments.

loop (data not shown). p53 did not bind to a control DNA duplex without triplet cytosines (see the *first panel* of Fig. 1). There was minimal DNA binding activity detected with control immunoprecipitates from yeast and Sf9 extracts lacking p53 protein (Fig. 1, see *Protein Con lanes*). The $p53^{273}$ tumor-derived mutant form of p53 was also tested in the assay and did not exhibit significant binding to DNA fragments containing IDLs.

Tumor-derived Mutant Forms of p53 Lack C_3 Binding Activity—The lack of IDL binding by the $p53^{273}$ protein prompted us to screen other p53 proteins with point mutations in the central domain. Four mutant p53 proteins representative of tumor-derived forms were produced in yeast and immunopurified, and equivalent amounts of protein were analyzed in the binding assay (Fig. 2*B*). We found that all four mutant p53 proteins had <5% of the wt p53 binding activity to the C_3 IDL (Fig. 2*A*). These data suggest that full-length p53 requires an intact central domain to bind DNA fragments containing IDLs. However, these results do not rule out the possibility that the p53 C terminus, as a separate entity, can also bind IDLs, as previously reported by Lee *et al.* (31).

Deletion of the C-Terminal 40 Amino Acids of p53 Abrogates C_3 Binding—To study the role of the p53 C terminus in IDL binding, engineered proteins with successive deletions of the C terminus were overexpressed in yeast, and equivalent amounts of proteins were tested for C_3 DNA fragment binding (Fig. 3*B*). Up to 40 amino acids could be deleted from the C terminus of p53 without loss of protein binding to IDLs (Fig. 3*A*; see *1–353*). In fact, deletion of 20 amino acids from the C terminus increased binding activity by more than 2-fold over wt p53 (Fig. 3*A*; see *1–373*). However, deletions of 50 or 60 amino acids resulted in 90% loss of IDL binding (Fig. 3*A*; see *1–343* and *1–333*).

The loss of IDL binding observed with deletions of 50 or more amino acids from the C terminus suggested that either the intrinsic C-terminal sequence was required for IDL binding or the oligomerization domain, which is encompassed within residues 312–365 of human p53 protein (37), must be intact for this p53 activity. To determine which of these two properties was necessary for p53-IDL binding, we used chimeric proteins containing various C-terminal-truncated p53 fused to the coil-coil (CC) dimerization domain of the yeast transcription factor GCN4 (residues 249–281) (38) for further analyses (Fig. 4*A*). p53-CC fusion proteins with deletions up to 100 amino acids from the p53 C terminus were able to bind IDLs (Fig. 4*B*). In fact, the p53-CC fusion proteins 293CC, 323CC, 333CC, 343CC displayed binding activities ~2–4-fold greater than wt protein. However, despite the enhanced binding seen with these fusion proteins, a single point mutation in the central domain of the p53 portion of the chimeric protein resulted in 90% loss of IDL binding (Fig. 4*A*, compare 343CC to $347CC^{175}$). Fusion proteins with p53 C-terminal deletions greater than 100 amino acids (273CC and 283CC) had less than 10% wt binding activity; these p53 deletions disrupted the central DNA binding domain, which is encompassed by residues 100–300 (39). Thus, the data suggest that full-length p53 binding to IDLs requires an intact

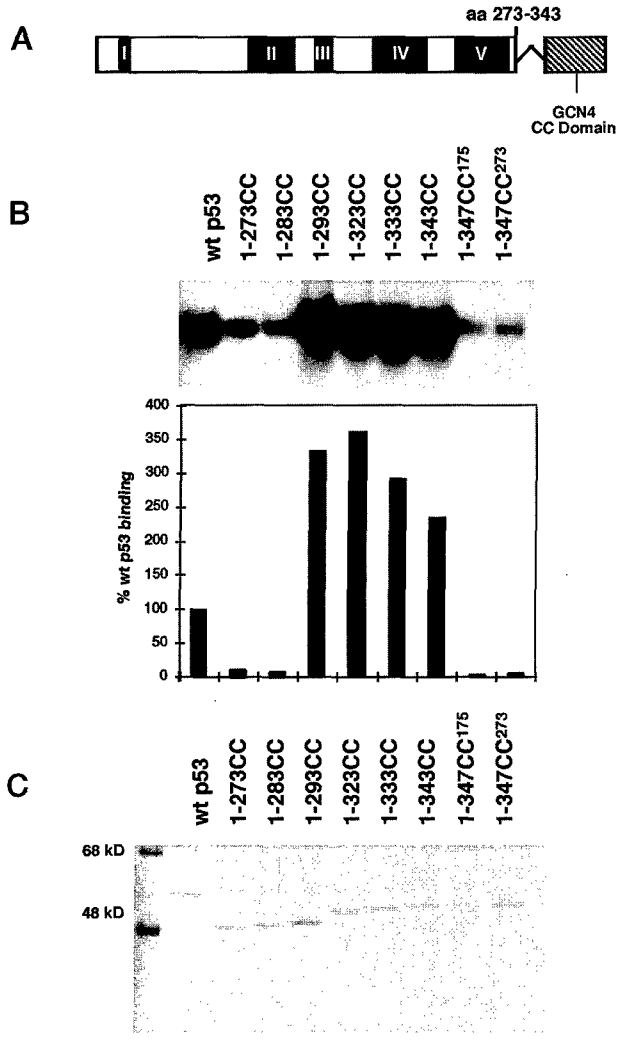

FIG. 4. **The dimerization domain of p53 can be replaced by the coiled-coil domain of the yeast transcription factor GCN4.** *A*, schematic representing the construction of C-terminal deletions of p53 fused to the CC dimerization domain of the yeast transcription factor GCN4. *I-V* represent evolutionarily conserved regions in p53. *aa*, amino acids. *B*, the results of binding assays performed with the C_3 DNA fragment and protein immunopurified from yeast cells engineered to express the indicated proteins. Quantification of the bound radiolabeled DNA is presented in the histogram. *C*, level and purity of the proteins used in the assay as visualized on a Coomassie Blue-stained SDS-polyacrylamide gel. Results are representative of five independent experiments.

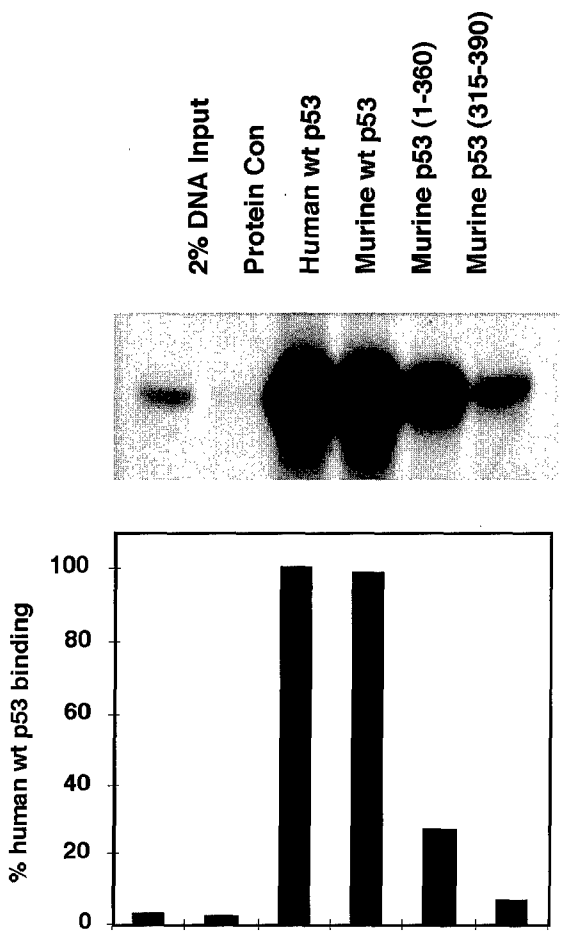

FIG. 5. **C_3 binding activity of full-length p53 and the C-terminal fragment.** The results of binding assays performed with the C_3 DNA fragment and protein immunopurified from a crude extract of Sf9 cells engineered to express the indicated proteins. Quantification of the bound radiolabeled DNA is presented in the histogram. Results are representative of three independent experiments. *Protein Con*, protein control.

central domain and that the entire C terminus of p53 is functionally replaceable with a foreign dimerization domain in these assays

C_3 Binding Activity of Full-length p53 Versus the C-terminal Fragment—Previous studies have reported that the C terminus alone can bind IDLs (31). To determine the relative binding activities of full-length p53 and the C-terminal fragment, we assayed the C_3 binding properties of wt human p53 and three forms of murine p53 produced in Sf9 cells: full-length p53, amino acids 1–360, and amino acids 315–390 (40). Binding assays were performed with equimolar amounts of p53 protein incubated with the C_3 DNA fragment (Fig. 5). Murine and human wt p53 had equal C_3 binding activity. Similar to our results with human p53 C-terminal truncation mutants (Fig. 3*A*), partial deletion of the C terminus of murine p53 significantly lowered C_3 binding ability. Consistent with the results of Lee *et al.* (31), we found that the C-terminal fragment of p53 could bind C_3, albeit with less than 10% full-length p53 IDL binding activity.

Saturation Binding of p53 to DNA Fragments Containing Consensus Sites or IDLs—The significance of p53 binding to its consensus DNA site is supported by many biological studies (41) and the report of nM affinities for p53 binding to derivatives of its consensus site (42). The lack of significant C_3 binding by tumor-derived mutant forms of p53 suggests this activity, like the p53 consensus binding, may be biologically relevant. To determine whether IDL binding occurs in a physiologic range, we performed saturation binding assays to compare the affinity of full-length, wt human p53 for its consensus site with that for C_3. The p53 binding site in the p21 promoter (nucleotides 2293–2332) was used for consensus binding analyses. Using 0.1 pmol of p53 protein and increasing amounts (0.5 to 750 pM) of labeled DNA, we determined that the K_D for p53 binding to its consensus site was 31 pM (Fig. 6*A*), and the K_D for p53 binding to the C_3 DNA was 45 pM (Fig. 6*B*). Transformation of the saturation binding isotherms into linear Scatchard plots facilitates the comparison of p53 binding to the two DNA fragments (Fig. 6*C*). Although the binding affinity and capacity of p53 for its consensus site are higher than those for C_3, both K_D values represent physiologically significant binding.

Competition of p53 DNA Binding—To confirm the relative affinities determined using saturation binding curves and to study the DNA binding property of p53 when both a consensus DNA site and IDL DNA site were available, competition binding assays were performed. Under equilibrium conditions, p53

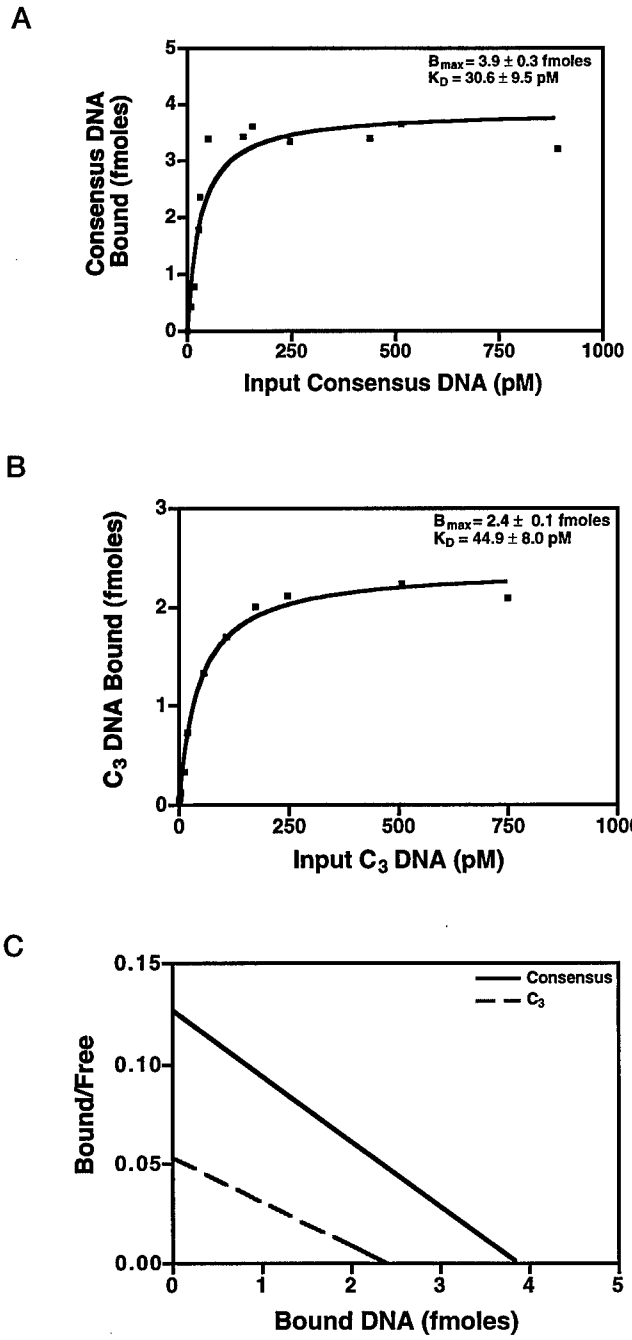

FIG. 6. **p53 affinity for DNA fragments containing IDLs.** Binding assays were performed by incubating 0.1 pmol of p53 with increasing amounts of either *A*, p53 consensus site representing nucleotides 2293–2332 of the p21 promoter; or *B*, C_3 DNA fragment. K_D represents the concentration of p53 required to reach half-maximal binding of the DNA. B_{max} is the maximal binding. The equation used to plot the data represents a rectangular hyperbolic function, indicative of binding that follows the mass action law: $Y = B_{max} \times X/(K_D + X)$. Scatchard analysis was perfomed on the saturation-binding isotherms, and the linear representation of the data is seen in *panel C*. Results are representative of three independent experiments.

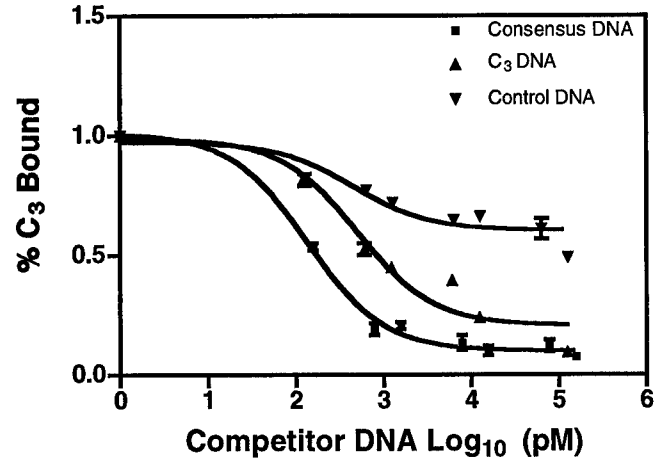

FIG. 7. **Dissociation of p53-IDL complexes by competitor DNA.** Competition assays were performed at equilibrium binding conditions of p53 binding to the C_3 fragment. After binding the ^{32}P-end-labeled C_3 DNA fragment, increasing concentrations of either unlabeled control, consensus site, or C_3 DNA fragments were incubated with the complex. EC_{50} represents the concentration of competitor that reduced p53-DNA binding by 50%. Competition assays were analyzed using GraphPad Prism software. The equation used to plot the data is indicative of competition of binding to a single site: Y = Bottom + (Top-Bottom)/(1 + $10^{(X - EC50)}$).

was first bound to the radiolabeled C_3 DNA fragment, and increasing concentrations of either unlabeled consensus site, C_3, or a random sequence dsDNA fragment were added. p53 was efficiently competed off of C_3 after addition of consensus site DNA with an EC_{50} of 0.14 nM (Fig. 7). The EC_{50} for the C_3 DNA fragment competition of prebound C_3 DNA was 0.50 nM (Fig. 7). In contrast, a random sequence dsDNA fragment was an ineffective competitor for C_3 binding. The EC_{50} for this latter competition assay could not be accurately assessed because 50% competition was difficult to attain (Fig. 7). Also, in these assays, ssDNA was not an effective competitor as compared with C_3 (data not shown). These data demonstrate that p53 has a higher affinity for its consensus site as compared with an IDL site and will likely bind to the former if both DNA sites are present, with all other variables remaining constant.

DISCUSSION

In this study, we analyzed p53 binding to DNA fragments containing IDLs. We found that full-length p53 requires an intact central domain and dimerization capability to bind IDLs. Tumor-derived mutant forms of p53 lost IDL binding, and C-terminal truncation mutants still bound IDLs if the protein maintained dimerization capability. The pM dissociation constant that we observed for p53 binding to IDLs provides evidence for potential *in vivo* relevance.

Previously, the C terminus of p53 (amino acids 311–393) was shown to bind IDLs (35). Reed *et al.* (43) also find that the C terminus of p53 binds nonspecifically to dsDNA (43). These data contribute to a model suggesting that the p53 C terminus negatively regulates the sequence-specific binding activity of the protein. When the p53 C-terminal end is deleted, phosphorylated, acetylated, or bound to antibody, peptide, or single-stranded DNA, p53 binding to its consensus site is stimulated (18, 24, 34, 44). Although we demonstrated that the C terminus of murine p53 could bind IDLs, an equimolar amount of murine p53 lacking the C terminus had greater affinity for IDLs than the C-terminal fragment alone.

Our finding that the C terminus of p53 is not required for IDL binding is in agreement with the study of Parks *et al.* (45) demonstrating that p53 binding to IDLs was unaffected by PAb421, a monoclonal antibody that binds the C-terminal amino acids 371–380. Also, Bakalkin *et al.* (28) suggest that the domain of p53 responsible for nonspecific DNA binding depends on the DNA substrate; the C terminus of p53 binds single-stranded ends of DNA, whereas the central DNA binding domain of p53 binds internal ssDNA segments (28). The identity of the p53 binding domain(s) for various forms of DNA damage may suggest distinct roles for p53 in DNA damage-signaling pathways or DNA repair.

Several reports suggest that p53 is an important determinant in nucleotide excision repair (NER). Using cells derived from patients with Li-Fraumeni syndrome, Ford and Hanawalt (46) show that the efficiency of global NER was dependent on p53 status. Compared with cells heterozygous for p53, homozygous mutant p53 cells exhibited global NER deficiency; however, transcription-coupled repair was unaffected by the p53 status. The ability of p53 to bind RPA (47) and subunits of TFIIH (48), both of which are essential components of NER, suggests that p53 may play a direct role in NER. These protein associations are among those that define similarities between p53 and XPA, the damage recognition and binding component of NER (49). XPA binds DNA cooperatively with RPA (50) and once complexed with DNA, recruits TFIIH to the site of damage (51). p53 binding to IDLs may also be stimulated by RPA. XPA can bind to various damage lesions (49), and we have observed p53 binding to DNA fragments containing a cholesterol adduct (data not shown), an artificial DNA lesion used in *in vitro* DNA repair assays (52).

Although we show that p53 has higher affinity for its consensus site as compared with a DNA lesion, the temporal availability of p53 consensus binding sites must be considered. Wu and Levine (53) have reported that the p21 gene is the first measurable target of p53 transactivation after high dose UV irradiation. However, p53-mediated induction of p21 gene expression does not occur until 2 to 5 h post-irradiation. In agreement with this result, using *in vivo* footprinting, Chin *et al.* (54) did not observe significant DNaseI cleavage protection of the p21 promoter until 2 h after exposure of cells to 20 gray of ionizing radiation. After exposure of cells to 1 gray of ionizing radiation, 2 to 8 double-strand breaks/genome have been shown to occur (55). In separate studies, Ji *et al.* reported that treatment of cells with malondialdehyde (an endogenous product of lipid peroxidation) resulted in the formation of 300 M_1G-DNA adducts/genome and subsequent elevation of p53 activity several hours later (56). Thus, immediately after exposure of cells to genotoxic agents, the number of DNA lesions in a cell would likely exceed accessible p53 consensus sites, and p53 binding to DNA lesions may occur. The pM dissociation constant that we report for p53 binding to IDLs is consistent with the physiological concentrations of both p53 and DNA lesions in the cell after DNA damage. However, once DNA repair is initiated, the availability of consensus sites would likely increase, and the higher affinity of p53 for these sites would shift the binding equilibrium.

Our understanding of the biochemical activities that are required for p53 tumor-suppressive activities has increased enormously in recent years. However, the role of DNA damage intermediates and the network of signaling pathways by which cells activate p53 in the overall response to DNA damage is not well defined. Determining if p53 is directly activated by DNA damage intermediates and understanding how the sequence-specific and sequence-nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how human cells respond to a wide range of DNA lesions.

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